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# Coregulation by Phenylacetyl-Coenzyme A-Responsive PaaX Integrates Control of the Upper and Lower Pathways for Catabolism of Styrene by *Pseudomonas* sp. Strain Y2

Teresa del Peso-Santos,[1] David Bartolomé-Martín,[1] Cristina Fernández,[2] Sergio Alonso,[3] José Luis García,[2] Eduardo Díaz,[2] Victoria Shingler,[4] and Julián Perera[1]*

*Department of Biochemistry and Molecular Biology, I, Facultad de Ciencias Biológicas, Universidad Complutense, 28040 Madrid, Spain[1]; Department of Molecular Microbiology, Centro de Investigaciones Biológicas, CSIC, 28040 Madrid, Spain[2]; The Burnham Institute for Medical Research, La Jolla, California 92037[3]; and Department of Molecular Biology, Umeå University, S-901 87 Umeå, Sweden[4]*

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**The $P_{styA}$ promoter of *Pseudomonas* sp. strain Y2 controls expression of the *styABCD* genes, which are required for the conversion of styrene to phenylacetate, which is further catabolized by the products of two *paa* gene clusters. Two PaaX repressor proteins (PaaX1 and PaaX2) regulate transcription of the *paa* gene clusters of this strain. In silico analysis of the $P_{styA}$ promoter region revealed a sequence located just within *styA* that is similar to the reported PaaX binding sites of *Escherichia coli* and the proposed PaaX binding sites of the *paa* genes of *Pseudomonas* species. Here we show that protein extracts from some *Pseudomonas* strains that have *paaX* genes, but not from a *paaX* mutant strain, can bind and retard the migration of a $P_{styA}$ specific probe. Purified maltose-binding protein (MBP)–PaaX1 fusion protein specifically binds the $P_{styA}$ promoter proximal PaaX site, and this binding is eliminated by the addition of phenylacetyl-coenzyme A. The sequence protected by MBP-PaaX1 binding was defined by DNase I footprinting. Moreover, MBP-PaaX1 represses transcription from the $P_{styA}$ promoter in a phenylacetyl-coenzyme A-dependent manner in vitro. Finally, the inactivation of both *paaX* gene copies of *Pseudomonas* sp. strain Y2 leads to a higher level of transcription from the $P_{styA}$ promoter, while heterologous expression of the PaaX1 in *E. coli* greatly decreases transcription from the $P_{styA}$ promoter. These findings reveal a control mechanism that integrates regulation of styrene catabolism by coordinating the expression of the styrene upper catabolic operon to that of the *paa*-encoded central pathway and support a role for PaaX as a major regulatory protein in the phenylacetyl-coenzyme A catabolon through its response to the levels of this central metabolite.**

Aerobically, phenylacetic acid (PA) is catabolized through phenylacetyl-coenzyme A (PA-CoA), which is the common intermediate of the large PA-CoA catabolon (23). The *paa* gene clusters that encode the enzymes responsible for PA catabolism (Fig. 1) are widely distributed in bacteria (1, 4, 15, 26, 27, 30, 32). GntR-type PaaX repressors that bind to specific operator sites of the cognate promoter regions control transcription of the *paa* gene clusters. The interaction of PaaX with PA-CoA leads to its release from the operator sequence to allow transcription (14). More recently, PaaX has also been reported to act on the expression of the *pac* gene of *Escherichia coli*. This gene encodes the industrially useful penicillin G acylase, which appears to play a fundamental role in the PA-CoA catabolon by acting as a PA scavenger (17, 20).

Channeling of styrene to PA by means of the styrene upper catabolic pathway encoded by the *styABCD* operon (Fig. 1) has been documented for several *Pseudomonas* species (5, 28, 29, 31, 38). A typical two-component regulatory system comprising the StyS sensor kinase and the StyR DNA-binding response regulator is necessary for transcription from the $P_{styA}$ promoter of the *styABCD* operon (29, 31, 38). In the presence of styrene, StyS catalyzes the phosphorylation of StyR, which forms a dimer that recognizes three operator sites in the $P_{styA}$ promoter region (21, 22). This promoter region also contains a putative site for the binding of the integration host factor (IHF) (22, 33, 38).

*Pseudomonas* sp. strain Y2, a strain able to degrade styrene via the *sty* pathway (*styABCD*) (38), contains two functional copies of the *paa* gene cluster, named *paa1* and *paa2* (1, 4), that serve as the styrene lower catabolic pathways. Therefore, this strain also contains two copies of the PaaX regulator, designated PaaX1 and PaaX2, which share 86% identity (1, 4). In this work, we present in vivo and in vitro evidence that the regulation of the $P_{styA}$ promoter in *Pseudomonas* sp. strain Y2 is more complex than previously envisioned, since the PaaX regulator also binds to this promoter to repress transcription in the absence of PA-CoA. This finding expands our current view of the PaaX regulon to include the catabolism of toxic aromatic compounds, such as styrene, and places the PaaX protein at the center of an interesting case of an integrated regulatory strategy for the catabolism of aromatic compounds (reviewed by Shingler [34]), namely, a mechanism in which transcriptional control of the expression of the catabolic genes integrates responses to both a substrate (styrene through StyR/StyS) and a pathway intermediate (PA-CoA through PaaX). Conceptually, this regulatory scheme is reminiscent of that reported for the sub-

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, I, Facultad de Ciencias Biológicas, Universidad Complutense, 28040 Madrid, Spain. Phone: 34-913944145. Fax: 34-913944672. E-mail: perera@bio.ucm.es.

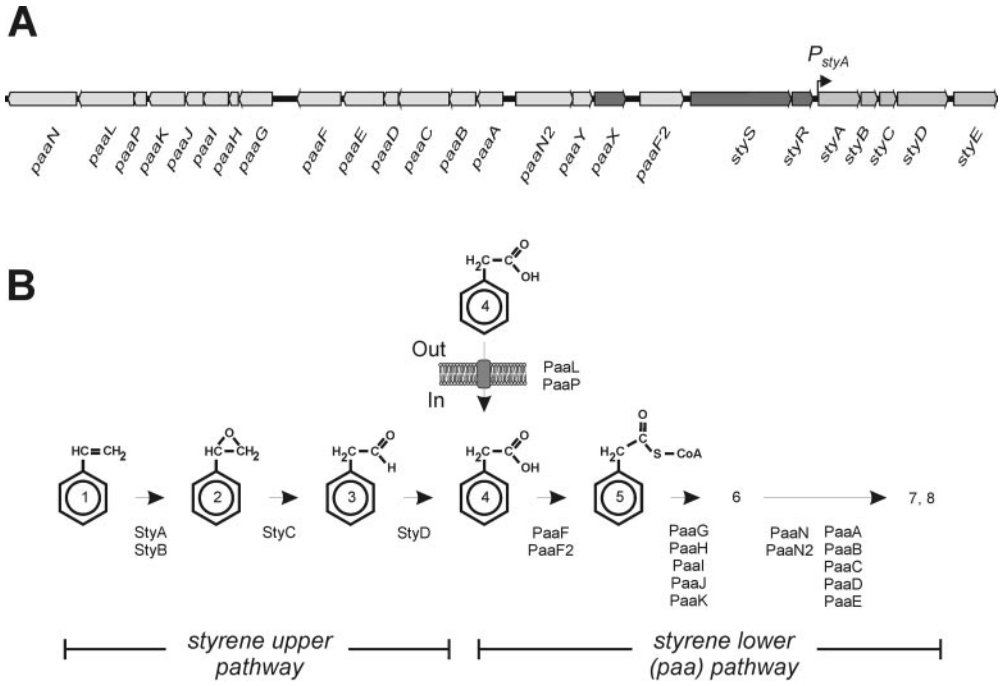


FIG. 1. Styrene catabolic genes and enzymatic pathway of *Pseudomonas* sp. strain Y2. (A) Genetic map of the *sty* and *paa1* gene clusters of *Pseudomonas* sp. strain Y2. The $P_{styA}$ promoter controlled by the StySR proteins regulates expression of the *sty* catabolic genes (*styABCD*) that encode the enzymes responsible for the transformation of styrene to phenylacetate; *styE* encodes a putative porin (38). The *paaF2N2ABCDEFGHIJKPLN* genes encode the enzymes that transform phenylacetate into metabolites of the Krebs cycle; *paaXY* genes encode regulatory proteins. The consensus nomenclature for the *paa* genes proposed by Luengo et al. (23) has been used. Arrows indicate the direction of transcription (1). (B) Styrene upper and lower catabolic pathways. Compounds are as follows: 1, styrene; 2, styrene oxide; 3, phenylacetaldehyde; 4, phenylacetate; 5, phenylacetyl-CoA; 6, hydroxy derivative of phenylacetyl-CoA; 7, aliphatic derivative of phenylacetyl-CoA; 8, intermediate metabolites of tricarboxylic acids cycle. Enzymes and proteins are as follows: StyAB, styrene monooxygenase; StyC, epoxystyrene isomerase; StyD, phenylacetaldehyde dehydrogenase; PaaL, permease; PaaP, membrane protein; PaaF and PaaF2, phenylacetyl-CoA ligases; PaaGHIJK, putative multicomponent phenylacetyl-CoA oxygenase; PaaN, PaaN2 and PaaABCDE, putative enzymes involved in ring-cleavage- and β-oxidation-like reactions of the aliphatic-CoA intermediate. "Out" and "in" refer to the periplasmic and cytoplasmic spaces, respectively (1, 4).

strate benzoate and intermediate *cis*-muconate in control of benzoate catabolism through the β-ketoadipate pathway (7, 8).

## MATERIALS AND METHODS

**Strain and plasmid constructions.** Bacterial strains, plasmids, and sequences of primers used in DNA manipulations are listed in Tables 1 and 2. Plasmids used for expressing the *paaX1* gene of *Pseudomonas* sp. strain Y2 under the control of the $P_{tac}$ promoter of different vectors were generated as follows. The *paaX1* gene, amplified from pUE14 using primers X5 and X3, was digested with EcoRI to XbaI and the resulting 1,049-bp fragment cloned between the corresponding sites of pSJP18Not to give rise to pJ18X1. The broad-host-range pVLTX1 plasmid carrying $P_{tac}$-*paaX1* was generated by cloning the *paaX1* gene excised from pJ18X1 as an EcoRI-to-HindIII fragment between the corresponding sites of pVLT31. As an intermediate in construction of a *paaX1* deletion mutant, the 1,046-bp EcoRI-to-SalI *paaX1* gene fragment from pJ18X1 was cloned between the same sites of pUCP26 to give plasmid pUXES. For expression of the maltose-binding protein (MBP)–PaaX1 fusion protein, the *paaX1* gene was PCR amplified from pUE14 plasmid by using primers BluntX5 and X3 and a 956-bp XbaI product cloned into XmnI to XbaI sites of pMAL-c2X vector to generate pM-X1.

*Pseudomonas* sp. strain Y2T2X1, which harbors a kanamycin resistance ($Km^r$) cassette replacing an internal portion of the *paaX1* gene of *Pseudomonas* sp. strain Y2T2, was generated by recombination as follows. A 1,252-bp HincII kanamycin-resistant fragment was isolated from pUC4K (Table 1) and cloned in the HincII site of pBluescript II KS(+) to give pBlueKm that allows recovery of the $Km^r$ cassette as a 1,296-bp EcoRV-KpnI fragment. The EcoRV-KpnI $Km^r$ cassette was used to replace the internal 499-bp SmaI-to-KpnI *paaX1* fragment of pUXES, yielding pUXKm. Primers BluntB5 and X3 (Table 2) were used to PCR amplify the *paaX1*-$Km^r$ deletion/replacement of pUXKm as a BamHI-to-XbaI fragment that was cloned between these sites of R6K-based suicide vector pKNG101 to give pKXKm in the replication permissive *E. coli* S17-1 λ*pir* strain. Finally, the mobilizable pKXKm suicide plasmid was conjugated to *Pseudomonas* sp. strain Y2T2 as previously described (1). Plating on kanamycin, tetracycline, and sucrose-containing medium was used as the method of selection for double recombinants, which were verified by screening for loss of the suicide plasmid by testing sensitivity to streptomycin and by PCR (data not shown) using BluntX5 and X3 primers (Table 2).

The in vitro transcription plasmid pTE-E1B1 was generated using primers E1 and B1 that amplify the −148-to-+67 $P_{styA}$ region (Fig. 2) as an EcoRI-to-BamHI fragment that was cloned between these sites of pTE103. Supercoiled DNA was prepared by CsCl gradient, extensively dialyzed, and clarified through Micro Bio-Spin P30 columns (Bio-Rad) equilibrated with 20 mM Tris-HCl, pH 7.5, to remove trace CsCl prior to use in vitro transcription assays.

**Crude extracts and purification of MBP-PaaX1 and His-StyR.** Crude extracts were prepared from *Pseudomonas* strains grown at 30°C in M9 minimal medium supplemented with 0.4% (wt/vol) glycerol and harvested at optical density at 600 nm ($OD_{600}$) of 1.0. Cells were washed and resuspended in 0.05 volumes of 20 mM Tris-HCl, pH 7.5, 10% glycerol, 2 mM β-mercaptoethanol, and 50 mM KCl prior to disruption by sonication. Crude extracts were clarified by removal of cell debris by centrifugation at 5,000 rpm for 45 min at 4°C and protein concentration was determined by the method of Bradford (6), using bovine serum albumin as the standard.

The MBP-PaaX1 fusion protein expressed by *E. coli* UT5600 harboring pM-X1 was purified through amilose chromatography columns as described in the pMAL protein fusion and purification system protocol (New England Biolabs) and conserved in the elution buffer (20 mM Tris-HCl, pH 7.5, 250 mM KCl, 15 mM maltose). Expression of the His-StyR fusion protein in *E. coli* MV1184 cells carrying plasmid pQ-R was induced by culturing in the presence of 1 mM

TABLE 1. Bacterial strains and plasmids

| Bacterial strain or plasmid | Relevant genotype/phenotype[a] | Reference/source |
|---|---|---|
| Strains | | |
| *Pseudomonas* sp. strain Y2 | *paa*$^+$ *sty*$^+$ *lacZ* Ap$^r$ Cm$^r$ | 37 |
| *Pseudomonas* sp. strain Y2T2 | Δ*paa2* deletion mutant of *Pseudomonas* sp. strain Y2; *paa*$^+$ *sty*$^+$ Ap$^r$ Cm$^r$ Tc$^r$ | 4 |
| *Pseudomonas* sp. strain Y2K1 | Δ*paa1* deletion mutant of *Pseudomonas* sp. strain Y2; *paa*$^+$ *sty*$^+$ Ap$^r$ Cm$^r$ Km$^r$ | 4 |
| *Pseudomonas* sp. strain Y2K1T2 | Δ*paa1* Δ*paa2* double deletion mutant of *Pseudomonas* sp. strain Y2; *paa sty* Ap$^r$ Cm$^r$ Km$^r$ Tc$^r$ | 4 |
| *Pseudomonas* sp. strain Y2T2X1 | Δ*paaX1* mutant of *Pseudomonas* sp. strain Y2T2; *paa*$^+$ *sty*$^+$ Ap$^r$ Cm$^r$ Tc$^r$ Km$^r$ | This work |
| *Pseudomonas putida* KT2442 | *paa*$^+$ *sty* Ap$^r$ Cm$^r$ | 3 |
| *Pseudomonas putida* U | *paa*$^+$ *sty* Ap$^r$ Cm$^r$ | 30 |
| *E. coli* UT5600 | F$^-$ *ara-14 leuB6 secA6 lacY1 proC14 tsx-67* Δ(*ompT-fepC*)*266 entA403 trpE38 rfbD1 rpsL109 xyl-5 mtl-1 thi-1* | 11 |
| *E. coli* MV1184 | *ara* Δ(*lac-proAB*) *rpsL thi* (Φ80 *lacZ*Δ*M15*) Δ(*srl-recA*)*306*::Tn*10* (Tc$^r$) F′ [*traD36 proAB*$^+$ *lacI*$^q$ *lacZ*Δ*M15*] | 39 |
| *E. coli* S17-1 λ*pir* | *recA thi hsdRM*$^+$ RP4::2-Tc::Mu::Km::Tn*7*, λ*pir* lysogen, Tp$^r$ Sm$^r$ | 10 |
| *E. coli* W14 | Δ*paa* derivative of *E. coli* W | 16 |
| Plasmids | | |
| pISm7.0R | pUC19-based plasmid containing *stySR-styAB* genes of *Pseudomonas* sp. strain Y2 after $P_{tac}$; Ap$^r$ | 38 |
| pUE14 | pUC19-based plasmid carrying a chromosomal EcoRI fragment of *Pseudomonas* sp. strain Y2 that spans part of *paaA*, *paaN2*, *paaY*, *paaX*, *paaF2*, *styS*, *styR*, *styA*, *styB*, and part of *styC* gene; Ap$^r$ | 38 |
| pSJP18Not | *ori* pCK01 Cm$^r$ | 18 |
| pJ18X1 | *paaX1* inserted between XbaI and EcoRI sites of pSJP18Not; Cm$^r$ | This work |
| pVLT31 | *ori* RSF1010 *lacI*$^q$/$P_{tac}$ Tc$^r$ | 9 |
| pVLTX1 | *paaX1* inserted between the EcoRI and HindIII sites of pVLT31; Tc$^r$ | This work |
| pMal-c2X | *ori* ColE1 *rop malE lacZ*α *lacI*$^q$/$P_{tac}$ Ap$^r$ | New England Biolabs |
| pM-X1 | *paaX1* inserted between the XmnI and XbaI sites of pMal-c2X; Ap$^r$ | This work |
| pTE103 | Plasmid which carries a strong T7 transcriptional terminator downstream of a multicloning site | 12 |
| pTE-E1B1 | pTE103 carrying the $P_{styA}$ promoter; Ap$^r$ | This work |
| pQ-R | pQE-9 (QIAGEN)-based plasmid carrying the *Pseudomonas* sp. strain Y2 *styR* gene fused to a amino-terminal polyhistidine (six-His) tag | S. Alonso |
| pUC4K | pUC4 derivative containing a Km resistance cassette; *ori* ColE1 Ap$^r$ Km$^r$ | 35 |
| pBluescript II KS(+) | General purpose cloning vector; *ori* ColE1 Ap$^r$ | Stratagene |
| pBlueKm | pBluescript II KS(+) with Km$^r$ cassette from pUC4K | This work |
| pUCP26 | Broad-host-range $P_{tac}$ cloning vector; *lacZ*α Tc$^r$ | 40 |
| pUXES | *paaX1* inserted in pUCP26; Tc$^r$ | This work |
| pUXKm | Δ*paaX1*-Km$^r$ gene deletion/replacement cassette on pUCP26; Km$^r$ Tc$^r$ | This work |
| pKNG101 | Mobilizable suicide vector for marker-exchange mutagenesis; *ori* R6K Sm$^r$ *sacB* | 19 |
| pKXKm | Δ*paaX1*-Km$^r$ gene deletion/replacement cassette on pKNG101; Km$^r$ Sm$^r$ *sacB* | This work |

[a] Ap$^r$, ampicillin resistant; Cm$^r$, chloramphenicol resistant; Tc$^r$, tetracycline resistant; Km$^r$, kanamycin resistant; Tp$^r$, trimethoprim resistant; Sm$^r$, streptomycin resistant.

IPTG (isopropyl-β-D-thiogalactoside) for 6 h. The fusion protein was purified from crude extracts prepared in lysis buffer (20 mM potassium phosphate, pH 7.5, 500 mM NaCl) by affinity chromatography using a Chelating Sepharose Fast Flow matrix (Amersham Biosciences) previously charged with $NiCl_2$ and equilibrated with lysis buffer. After washing with lysis buffer containing 75 mM imidazole, the His-StyR protein was eluted with lysis buffer containing 500 mM imidazole and buffer exchanged to 20 mM Tris-HCl, pH 7.5, 250 mM KCl, 5% glycerol using the Micrococon YM-10 centrifugal filter unit system (Millipore). Purified proteins were stored at −80°C.

**Electromobility shift and DNA footprinting assays.** The STY DNA probe was generated by PCR amplification using plasmid pISm7.0R and primers styAXL and styAXR (Fig. 2; Tables 1 and 2). Radioactivity was incorporated via the styAXL primer that was prelabeled by using [γ-$^{32}$P]ATP (>3,000 Ci/mmol) and T4 polynucleotide kinase. The resulting radioactive PCR product was purified using the High Pure PCR product purification kit (Roche). Electromobility shift reaction mixtures (9 μl final volume) containing 0.1 nM STY probe, 50 μg/ml salmon sperm DNA, the indicated amount of crude extract or purified MBP-PaaX1, and 50 μg/ml bovine serum albumin in T buffer (20 mM Tris-HCl, pH 7.5, 10% glycerol, 2 mM β-mercaptoethanol, 50 mM KCl) were incubated for 20 min at 20°C. The resulting complexes were analyzed by electrophoresis in 5% polyacrylamide gels buffered with 0.5× TBE (45 mM Tris-borate, 1 mM EDTA). The gels were dried onto Whatman 3MM filter paper and exposed to Hyperfilm MP (Amersham Biosciences).

DNase I footprinting reaction mixtures (15 μl final volume) were prepared and incubated with the indicated amount of MBP-PaaX1 as described above. Three microliters of DNase I (0.05 units DNase I from Amersham Biosciences prepared in 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 125 mM KCl) was added and reaction mixtures further incubated at 37°C for 20 to 25 s. The reactions were terminated by the addition of 180 μl of a solution containing 0.4 M sodium acetate, 2.5 mM EDTA, 50 μg/ml calf thymus DNA, and 0.3 μl/ml glycogen. After phenol-chloroform extraction, the samples were precipitated and washed with 70% ethanol, dried, and resuspended in 4 μl of 90% (vol/vol) formamide loading buffer (10 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.05% [wt/vol] bromophenol blue, 0.05% [wt/vol] xylene cyanol). Samples were denatured at 95°C for 5 min and analyzed on a 7% denaturing polyacrylamide-urea gel. A+G Maxam and Gilbert sequencing reactions (24) with the same DNA fragment were loaded along the footprinting samples, and the gels were treated as described above.

**In vitro transcription assays.** Reactions (20 μl final volume) were performed at 30°C in transcriptional buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 20 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, and 0.2 mg/ml bovine serum albumin. Purified His-StyR was phosphorylated just prior to in vitro-transcription assays by incubation at 28°C for 45 min in the same buffer supplemented to 20 mM $MgCl_2$ and 20 mM acetylphosphate. *E. coli* $\sigma^{70}$-RNA polymerase (Epicenter) (10 nM), the pTE-E1B1 template (5 nM), purified *E. coli* IHF (20 nM), phosphorylated His-StyR (2 μM), and the indicated amounts of purified MBP-PaaX1 and PA-CoA

TABLE 2. Primer sequences

| Oligonucleotide[a] | Sequence (5′→3′)[b] | Use |
|---|---|---|
| styAXR | TTTCAGGATATTTTTATACC | Amplification of STY probe |
| styAXL | CACTGTACTCATCGGGCTTA | |
| X5 | CGGAATTCGAAAGGCCAGGTGTGATG | Amplification of *paaX1* introducing an EcoRI restriction site at the 5′ end |
| X3 | GCTCTAGACCAGCAGCGGATTCGAG | Amplification of *paaX1* introducing an XbaI restriction site at the 3′ end |
| BluntX5 | ATGACGTCCCTCGCCCCACTGA | Amplification of *paaX1* |
| BluntB5 | CGCGGATCCGAAAGGCCAGGTGTGATG | Amplification of *paaX1* introducing a BamHI restriction site at the 5′ end |
| E1 | CGGAATTCGTTGACTGCTCCTG | Amplification of the *styS* to *styA* intergenic region introducing EcoRI (E1) and BamHI (B1) restriction sites |
| B1 | CGCGGATCCTGCCTGCACCA | |
| QstyA (F) | GAGCCTTGAGATCACCGTAGAAG | Quantification of *styA* mRNA levels by RT-PCR |
| QstyA (R) | TTTGGCTATTTCGGTCACTACTACTAC | |
| QTcR2 (F) | AATGCGCTCATCGTCATCCT | Quantification of *tetA* mRNA levels by RT-PCR |
| QTcR2 (R) | GGCAGTACCGGCATAACCAA | |
| ApL2 | ATCCGCCTCCATCCAGTCTA | Quantification of *bla* mRNA levels by RT-PCR |
| ApR2 | AACAACGTTGCGCAAACTATT | |

[a] F, forward; R, reverse.
[b] Restriction sites introduced in the amplification product by use of the coresponding primer are underlined.

were mixed and incubated for 15 min to allow open complex formation. Multiple-round transcription was initiated by adding a mixture of 500 μM ATP, 200 μM GTP, 200 μM CTP, 80 μM UTP, and 5 μCi of [α-$^{32}$P]UTP (>3,000 Ci/mmol; Amersham Biosciences). After 5 min of incubation, reinitiation was prevented by the addition of heparin (0.1 mg/ml), and 5 min later, the reactions were terminated by adding 5 μl of stop/loading buffer (150 mM EDTA, 1.05 M NaCl, 14 M urea, 10% glycerol, 0.037% xylene cyanol, 0.037% bromophenol blue). Samples were analyzed on a 7 M urea–5% polyacrylamide sequencing gel and quantified using an Amersham Biosciences phosphorimager. Transcript production was normalized to the production of the RNA-1 transcript from the pTE plasmid vector.

**Quantitative RT-PCR.** For RNA isolation, *Pseudomonas* strains were grown in M9 minimal medium (25) with 0.8% (wt/vol) glycerol as the sole carbon and energy source until the culture $OD_{600}$ reached 0.6. *E. coli* cells were grown in M63 medium (25) with 0.4% (wt/vol) glycerol as the sole carbon and energy source until the culture $OD_{600}$ reached 0.4, and then IPTG (1 mM) was added to induce PaaX1 expression from the resident plasmid until cultures reached an

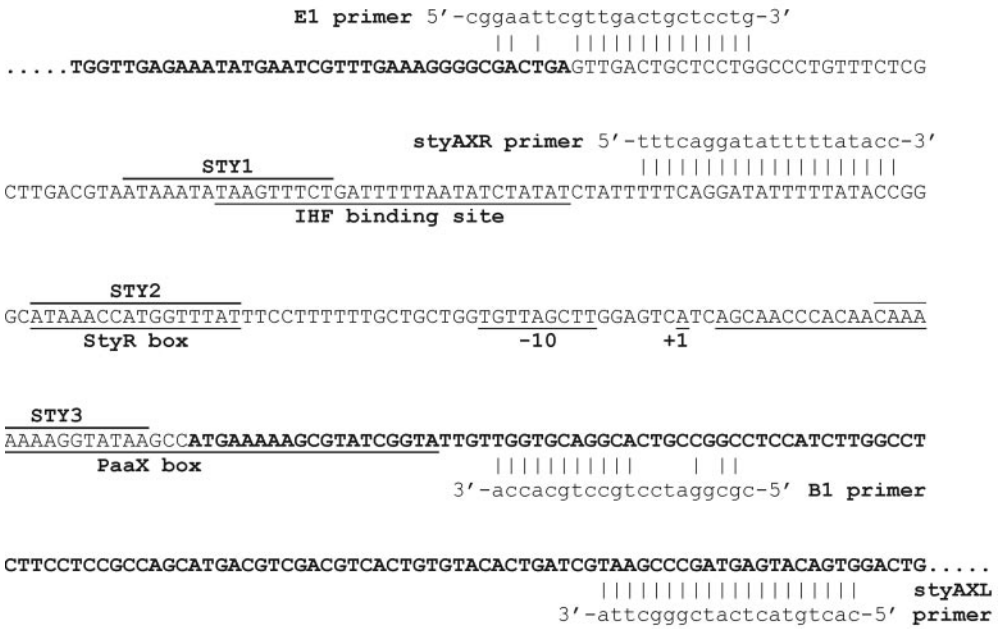


FIG. 2. Sequence of the $P_{styA}$ region of *Pseudomonas* sp. strain Y2. The 3′ end of *styR* and the 5′-end region of *styA* are in boldface letters. The StyR binding site proposed for *Pseudomonas* sp. strain Y2 (38) is underlined. The high- (STY2), medium- (STY1), and very-low-affinity (STY3) StyR binding sites of *P. fluorescens* ST proposed by Leoni et al. (22) are overlined. The IHF binding site, the extended −10 box of $P_{styA}$ promoter, the transcription start site (+1) for *styA*, and the PaaX binding site (PaaX box) are underlined. The locations and sequences of styAXR and styAXL oligonucleotides used to amplify the STY probe and the E1 and B1 oligonucleotides used to amplify the *styR-styA* intergenic region are indicated in lowercase letters.

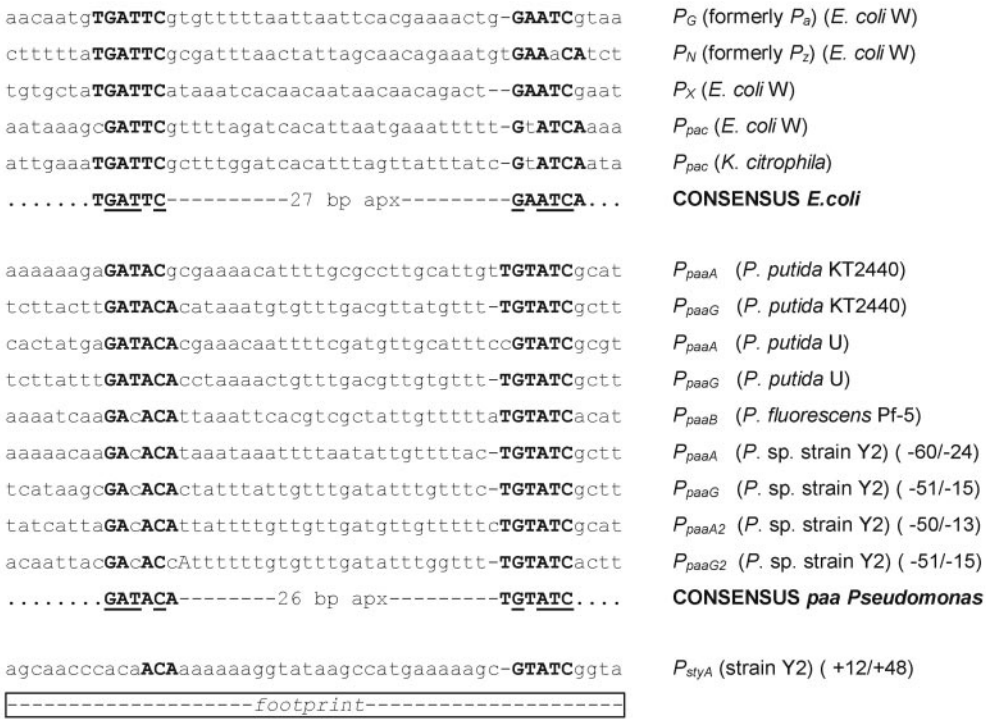


FIG. 3. PaaX binding sites. Comparison of PaaX binding sites of several promoters of *E. coli* W (GenBank accession number X97452) and *Kluyvera citrophila* (M15418) with putative PaaX binding sites found in the *paa* promoters of *P. putida* KT2440 (AE015451), *P. putida* U (AF029714), *P. fluorescens* Pf-5 (NC004129), and *Pseudomonas* sp. strain Y2 (AJ000330 and AJ579894) and with the PaaX binding site identified in $P_{styA}$. $P_G$ (formerly $P_a$), $P_N$ (formerly $P_z$), and $P_X$ refer to the promoters that control transcription of the three *paa* operons of *E. coli*. $P_{pac}$ is the promoter that drives the transcription of the penicillin G acylase gene. $P_{paaA}$, $P_{paaG}$, $P_{paaA2}$, $P_{paaG2}$, and $P_{paaB}$ are promoters of the corresponding *paa* genes of *Pseudomonas* species. $P_{styA}$ is the promoter that controls the transcription of the *styABCD* styrene catabolic operon of *Pseudomonas* sp. strain Y2. The location of the region encompassing both 6-bp inverted repeats relative to the putative transcription start point is given in parentheses at right. Consensus sequences of putative PaaX binding sites are also displayed. Nucleotides identical to the consensus are shown in uppercase bold letters. Conserved nucleotides between the *E. coli* and *Pseudomonas* consensus sequences are underlined. The region of $P_{styA}$ protected by PaaX1 of *Pseudomonas* sp. strain Y2 against DNase I is displayed as a box (see the text).

$OD_{600}$ of 0.6. At that point, each culture was divided into two flasks. One flask of every pair was induced with styrene supplied in gas phase via saturated atmosphere (*Pseudomonas* cultures for 5 min; *E. coli* cultures for 30 min), whereas the other was left uninduced. TRI reagent (Ambion) and RNAqueous kit (Ambion) were used to extract the total RNA from 12-ml culture samples according to the manufacturer's instructions. Agarose gel electrophoresis confirmed the integrity of the RNA. For cDNA synthesis, 2.5 μg of total RNA as determined spectrophotometrically was incubated for 3 h at 42°C with 200 U of SuperScript II reverse transcriptase (Invitrogen) in the presence of random hexamer primers. Control reactions to assess the level of DNA contamination in the RNA samples were carried out without reverse transcriptase. Once the reactions were completed, RNase A (Roche) and RNase H (USB) were added to the reaction mixtures to remove the remaining RNA. The synthesized cDNA was purified using a QIAquick PCR purification kit (QIAGEN), and its concentration was determined spectrophotometrically. Real-time PCR (RT-PCR) was performed using an ABI PRISM 7700 sequence detection system (Applied Biosystems) with SYBR green PCR master mix (Applied Biosystems). Master mixtures were prepared as recommended by the manufacturer, with primer concentrations optimized for each primer pair. The QstyA (F) and QstyA (R) primers (Table 2) amplify a 73-bp fragment of *styA*. Internal standards for normalization were provided by the QTcR2 (F) and QTcR2 (R) primers and the ApL2 and ApR2 primers (Table 2) that amplify a 74-bp fragment and an 82-bp fragment from the *tetA* (tetracycline resistance) and *bla* (ampicillin resistance) genes, respectively. For quantification, standard curves of cDNA dilutions (1:10 to 1:10,000) were made in duplicate for each primer pair. PCR efficiencies were similar, allowing direct comparison of results.

## RESULTS AND DISCUSSION

**The $P_{styA}$ promoter region of *Pseudomonas* sp. strain Y2 contains a putative binding site for PaaX.** Initially, a 15-bp consensus sequence was proposed as the binding site for PaaX at different *paa* promoters (14, 17), but this sequence was later extended to a 39-bp region with two perfect 6-bp inverted repeats (TGATTC and GAATCA) separated by 27 nucleotides (20) as shown in Fig. 3. An in silico analysis of the *paa* clusters of *Pseudomonas* sp. strain Y2, made using the Fuzznuc program from the Emboss suite (http://emboss.sourceforge.net/), revealed the existence of several putative PaaX binding sites very similar to putative PaaX binding sites proposed for homologous *paa* operons of other *Pseudomonas* species. The *Pseudomonas* consensus sequence for putative PaaX binding sites consists of two perfect inverted repeats (GATACA and TGTATC) about 26 bp apart, which bear some similarity to those reported for *E. coli* (Fig. 3). Given that PaaX operator sequences have also been found to function within promoters of non-*paa* genes (17, 20), we also performed an in silico analysis of the promoter region of the styrene upper catabolic genes of *Pseudomonas* sp. strain Y2. This analysis revealed a previously unidentified putative

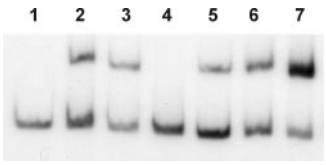


FIG. 4. Electrophoretic mobility shift assays of the STY probe with cell extracts from different *Pseudomonas* species. Lane 1, free STY probe; lane 2, cell extracts of *Pseudomonas* sp. strain Y2K1 (Δ*paa1*) (4.4 μg/μl); lane 3, cell extracts of *Pseudomonas* sp. strain Y2T2 (Δ*paa2*) (4.2 μg/μl); lane 4, cell extracts of *Pseudomonas* sp. strain Y2K1T2 (Δ*paa1* Δ*paa2*) (3.5 μg/μl); lane 5, cell extracts of *P. putida* KT2442 (2.3 μg/μl); lane 6, cell extracts of *P. putida* U (5.5 μg/μl); lane 7, cell extracts of *Pseudomonas* sp. strain Y2 (pVLTX1) (3.3 μg/μl).

PaaX binding site located between positions +12 and +48 with respect to the transcriptional start site from $P_{styA}$, just within the *styA* gene of the *styABCD* operon (Fig. 2 and 3). This putative site contains five of the six nucleotides present in the right-half inverted repeat of the consensus, but only the last three of the six consensus residues of the left hand repeat (Fig. 3, bottom panel). Although divergent from the consensus, this finding suggested to us that PaaX might bind the $P_{styA}$ promoter of *Pseudomonas* sp. strain Y2, adding previously unsuspected complexity to the regulation of this promoter.

**PaaX binds the $P_{styA}$ promoter region in vitro.** To explore the possibility that PaaX binds the $P_{styA}$ promoter region, we performed electrophoretic mobility shift assays using a STY DNA probe containing the putative PaaX binding site in the $P_{styA}$ promoter and cell extracts from different *Pseudomonas* strains that either contain or lack *paaX* genes. To avoid interferences by IHF in the assays with cellular crude extracts, the DNA fragment used (−73 to +154) (Fig. 2) lacks the binding site for IHF. As shown in Fig. 4, crude extracts from the *Pseudomonas* strains containing a single *paaX gene*, e.g., *Pseudomonas putida* KT2442, *P. putida* U, and the two *Pseudomonas* sp. strain Y2 deletion mutants, K1 (Δ*paa1*) and T2 (Δ*paa2*), that lack either *paaX1* or *paaX2*, were able to bind and retard the migration of the STY probe. However, crude extracts from *Pseudomonas* sp. strain Y2K1T2, a double *paa* deletion mutant (Δ*paa1* Δ*paa2*), did not retard migration of the STY probe (Fig. 4, lane 4). Interestingly, retardation of the STY probe increased significantly when using a crude extract from *Pseudomonas* sp. strain Y2 harboring pVLTX1 (Fig. 4, lane 7), which overexpresses PaaX1 from the $P_{tac}$ promoter of the plasmid. *P. putida* KT2442 and *P. putida* U lack the specific StyR regulator; therefore, these results suggest that the *paaX* gene products of different *Pseudomonas* strains might bind the $P_{styA}$ promoter and that both the *paaX1* and *paaX2* gene products of *Pseudomonas* sp. strain Y2 are capable of binding to the $P_{styA}$ proximal PaaX binding site (Fig. 2).

To confirm specific binding and to study the interaction between PaaX and the $P_{styA}$ promoter, we purified the PaaX1 regulator with an N-terminal fusion to the maltose-binding protein. The purified MBP-PaaX1 protein retards the STY DNA probe in a dose-dependent fashion (Fig. 5A), and retardation can be specifically blocked by the addition of cold competitor STY DNA (Fig. 5B, lane 9). Controls made with MBP alone do not retain the probe (data not shown). In *E. coli*, PA-CoA induces the transcription of PaaX-dependent promoters through a mechanism that involves the direct binding of PA-CoA to PaaX and consequent release of this repressor (14, 17, 20). Therefore, we analyzed whether a similar process might control binding of PaaX to the $P_{styA}$ proximal binding site. As shown in Fig. 5B, this appears to be the case, since MBP-PaaX1 binding to the STY DNA probe is inhibited by PA-CoA but not by PA (Fig. 5B). Thus, PA-CoA controlled binding of PaaX may play an integral role in regulation of the expression of *sty* genes.

**Characterization of the PaaX operator site within the $P_{styA}$ promoter region.** To define the limits of the sequence bound to PaaX1 in the $P_{styA}$ region, we performed a DNase I footprinting analysis. Consistent with the in silico analysis, MBP-PaaX1 protects a region spanning from positions +4 to +52 with respect to the transcriptional start site of the *styABCD* operon (Fig. 6). The +4-to-+52 region is hereafter referred to as the PaaX operator region (Fig. 2). Interestingly, this PaaX operator region is located just inside the open reading frame of *styA*. This contrasts the location of the putative PaaX operators proposed for the *paa* operons of *Pseudomonas* sp. strain Y2, which overlap the −35 box of the putative promoters (Fig. 3). In these instances, PaaX would presumably repress transcription by the common repressor mechanism of inhibiting RNA polymerase binding. In the case of the $P_{styA}$ PaaX operator, any effect of PaaX binding on transcription would have to operate by a different mechanism. The possibility that it could function through hindrance of the formation of the open transcription complex, as previously suggested for the homologous PaaX

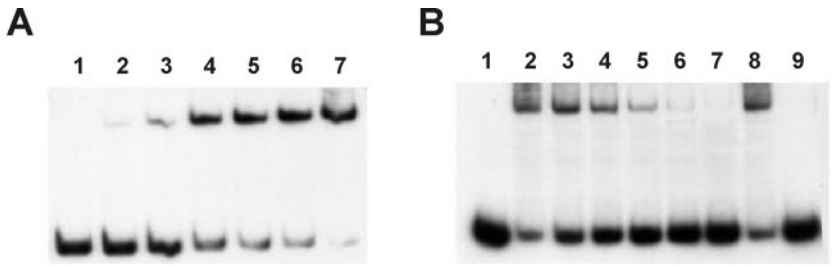


FIG. 5. Electrophoretic mobility shift assays of the STY probe with purified MBP-PaaX1 protein. (A). Lanes 1 to 7, retardation assays of the STY probe with 0, 5, 10, 25, 50, 100, and 150 nM MBP-PaaX1, respectively. (B). Lane 1, free STY probe. Lanes 2 to 7, retardation assays produced by 150 nM purified MBP-PaaX1 in the presence of 0, 25, 50, 100, 500, and 1,000 μM PA-CoA, respectively. Lane 8, retardation assay produced in the presence of 150 nM MBP-PaaX1 and 1 mM PA. Lane 9, retardation produced by 150 nM MBP-PaaX1 in the presence of 10 nM unlabeled STY probe.

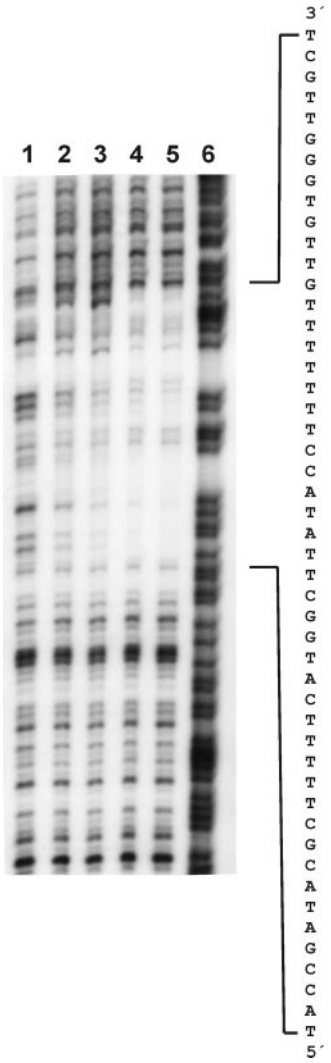


FIG. 6. DNase I footprinting analysis of the interaction of purified MBP-PaaX1 with the STY probe. Lanes 1 to 5 show footprints with 0, 25, 50, 150, and 300 nM purified MBP-PaaX1, respectively. Lane 6, A+G Maxam and Gilbert sequencing reaction. The nucleotide sequence of the protected region, complementary to that shown in Fig. 2 and 3, is shown within brackets.

repressor protein of *E. coli* (13, 36), will be a matter of further study.

**MBP-PaaX1 is a PA-CoA-responsive transcriptional repressor of transcription from $P_{styA}$ in vitro.** The location of the PaaX operator site and the PaaX-binding studies described above suggest that PaaX might act as a transcriptional repressor of the $P_{styA}$ promoter. To determine if this is the case in vitro, we constructed plasmid pTE-E1B1 that carries the $P_{styA}$ promoter region (−148 to +67) that includes the whole of the *styR* to *styA* intergenic region and the 5′ end of *styA* (Fig. 2). This template allows monitoring of StyR-dependent transcription from the $P_{styA}$ promoter (Fig. 7). In the absence of StyR, no *styA* transcript band was seen (data not shown). In the presence of phosphorylated StyR, the addition of increasing concentrations of MBP-PaaX1 causes a significant reduction in the transcript levels from the $P_{styA}$ promoter. Moreover, the presence of PA-CoA significantly reduces the repressive effect of MBP-PaaX1 on $P_{styA}$ transcription (Fig. 7B, inset). Thus, we conclude that, like PaaX-mediated regulation of the *paa* genes in *E. coli*, PaaX1 can serve as a PA-CoA-responsive repressor of the *Pseudomonas* sp. strain Y2 $P_{styA}$ promoter in vitro.

**PaaX1 represses both basal and styrene-induced $P_{styA}$ transcription in vivo.** The in vitro data described above show that PaaX1 has the potential to repress $P_{styA}$ transcription in the absence of PA-CoA and that this repression counteracts StyR activated transcription from the promoter. To see if PaaX1 also counteracts styrene-induced transcription in vivo, we first employed quantitative RT-PCR analysis of transcription from the $P_{styA}$ promoter using a two-plasmid system in the heterologous host *E. coli* W14. This host is a derivative of *E. coli* W that is unable to use PA as a carbon source (16) and hence unable to make PA-CoA from styrene added to induce transcription. The pISm7.0R plasmid carries $P_{lac}$-*stySR*-$P_{styA}$-*styAB* DNA and thus provides the $P_{styA}$ promoter in its native DNA context along with genetic components necessary for styrene-induced transcription (38). The coresident plasmid, either the vector control pSJP18Not or pJ18X1 that carries *paaX1* under the control of the $P_{lac}$ promoter, completes the genetic system to monitor the effects of PaaX1 on transcription. As shown in Fig. 8A, in the absence of PA-CoA, the PaaX1 protein can repress styrene-induced transcription from $P_{styA}$ since the levels of transcripts in *E. coli* W14(pISm7.0R)(pJ18X1) after 30 min of exposure to styrene are markedly lower than those of *E. coli* W14(pISm7.0R)(pSJP18Not).

To determine the in vivo consequences of the action of PaaX1 at the $P_{styA}$ promoter in its native host, we measured *styA* mRNA levels by using *Pseudomonas* sp. strain Y2 derivatives lacking either PaaX2 (*Pseudomonas* sp. strain Y2T2) or both PaaX1 and PaaX2 (*Pseudomonas* sp. strain Y2T2X1). Consistent with its role as a repressor in vitro (Fig. 7B), lack of PaaX1 in the native host also results in higher transcription from $P_{styA}$ after 5 min of exposure to styrene (Fig. 8B). Even in the absence of induction, the lack of PaaX1 allowed a three-times-higher basal transcription level (from 1.0 ± 0.18 to 3.02 ± 0.41). Both of these strains retain one *paa* gene cluster and are thus capable of converting styrene to the PA-CoA effector molecule that relieves MBP-PaaX1 binding (Fig. 5B) and transcriptional repression (Fig. 7B) in vitro. Hence, these results suggest that despite the presence of PA-CoA, native levels of PaaX1 still repress styrene-activated transcription.

PaaX2 is highly similar (86% identity) to PaaX1 and extracts expressing just PaaX2 can retard the STY DNA probe (Fig. 4, lane 2). Thus, it is probable that PaaX2 can act like PaaX1 to play a similar regulatory role at the $P_{styA}$ promoter. Notably, as mentioned above, the lack of both PaaX1 and PaaX2 also results in comparatively high noninduced basal levels of transcription from $P_{styA}$ (Fig. 8B). These results suggest that, in addition to modulating styrene-induced levels of transcription, PaaX1 and, by inference, PaaX2 may play an important regulatory role in maintaining tight regulation of $P_{styA}$ in the absence of substrate for the pathway it regulates.

**Concluding remarks.** This work reveals a previously unsuspected role for a PaaX protein in regulating expression of *sty* genes involved in styrene catabolism. The existence of a PaaX-binding site at the $P_{styA}$ promoter provides an additional level of transcriptional control that integrates transcription from the

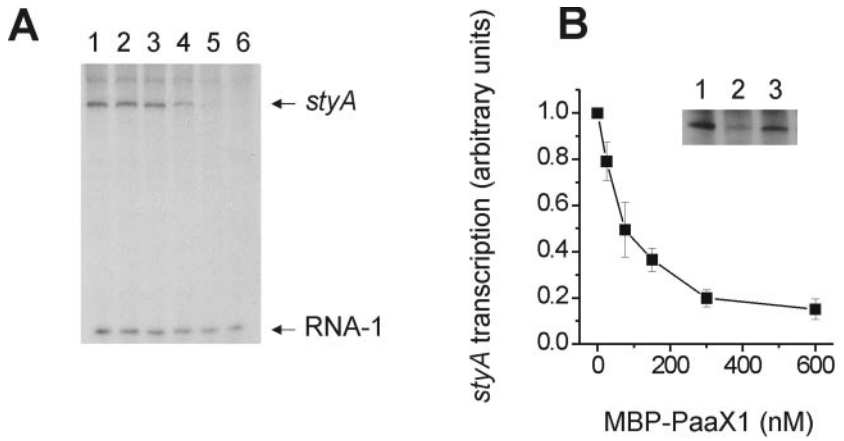


FIG. 7. Repression of in vitro transcription from $P_{styA}$ promoter by MBP-PaaX1 and effect of the presence of PA-CoA. (A). Transcripts from pTE-E1B1 in the presence of phosphorylated StyR and different amounts of MBP-PaaX1. Lane 1 to lane 6, 0, 25, 75, 150, 300, and 600 nM MBP-PaaX1, respectively. Arrows point to the *styA* transcript (*styA*) and to the internal control transcript (RNA-1). (B). *styA* transcript levels (in arbitrary units) produced from pTE-E1B1 template in the presence of 0 to 600 nM purified MBP-PaaX1. The results are the normalized averages of six independent experiments. Error bars, standard errors. The inset shows the autoradiography of *styA* transcripts produced in the absence of MBP-PaaX1 (lane 1), in the presence of 600 nM MBP-PaaX1 (lane 2), and in the presence of 600 nM MBP-PaaX1 challenged with 1 mM PA-CoA (lane 3).

$P_{styA}$ promoter, making it responsive to both initial styrene pathway substrate, through StySR, and the central intermediate PA-CoA produced by the *paa* genes, through the action of PaaX. Thus, this regulatory mechanism is likely to facilitate balanced and coordinated expression of the styrene upper (*sty* operon) and lower (*paa* genes) pathways and central metabolism. In this respect, it is interesting that inactivation of the *paaX* homologue of *P. putida* U (*phaN*) relieves the repression of the *paa* genes when this strain grows in the presence of glucose (30). The $P_{styA}$ proximal PaaX-binding site identified here overlaps the very-low-affinity StyR-binding site (STY3) recently described in the *sty* operon of *P. fluorescens* ST (22). In the *P. fluorescens* ST system, StyR has been postulated to behave as a transcriptional repressor when it binds to STY3, and this binding is involved in the glucose-mediated repression of $P_{styA}$. In agreement with this, mutations in the STY3 operator region in *P. fluorescens* ST, which corresponds to the PaaX-binding site in *Pseudomonas* sp. strain Y2, partially alleviates the glucose-mediated repression of the $P_{styA}$ promoter (22). While we cannot exclude the possibility that control of

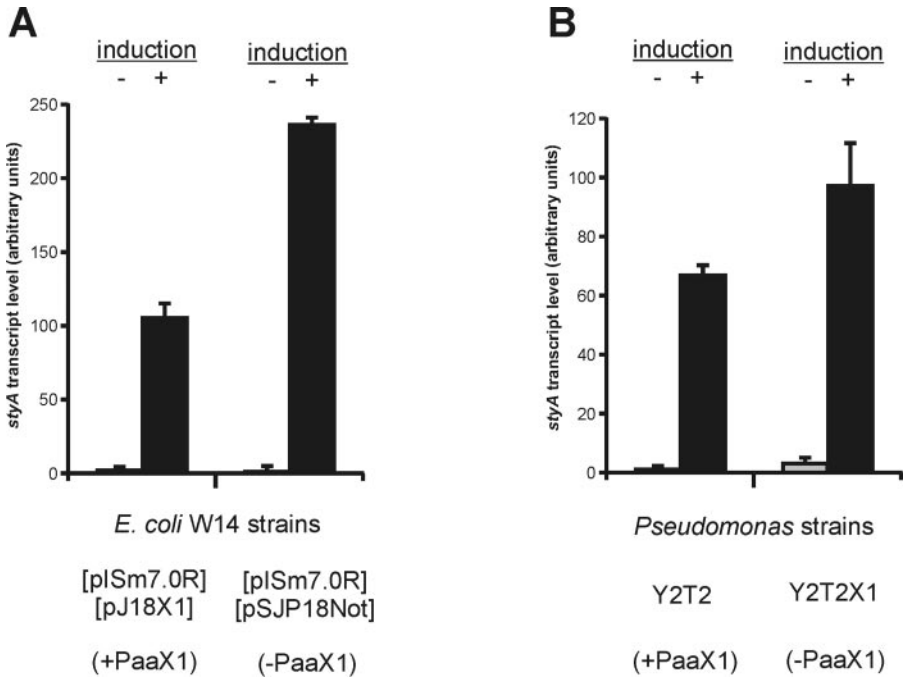


FIG. 8. RT-PCR quantification of *styA* mRNA levels in different *Pseudomonas* and *E. coli* strains. The *styA* transcript level was determined in cultures of the indicated strains grown under either noninduced (gray bars) or styrene-induced (black bars) conditions as described in Materials and Methods. Bars represent the means of *styA* transcript levels from three independent experiments, each performed in duplicate. Error bars, standard errors.

the *sty* genes in strains ST and Y2 might differ, our work offers an attractive alternative interpretation, namely, that PaaX binding, rather than StyR binding, to this region controls glucose-mediated catabolite repression of the $P_{styA}$ promoter. Direct analysis of this putative role for the PaaX regulator is the subject of further research.

The work described here expands the known PaaX regulon to include the *styABCD* operon and supports the notion that PaaX, by sensing the PA-CoA, is a master regulatory protein in the PA-CoA catabolon that adjusts the expression of different operons to that of the *paa*-encoded central pathway (17). PaaX-mediated repression of the $P_{styA}$ promoter acts in concert with the specific StyS-StyR regulatory system to provide a dual signal-responsive system that is functionally analogous to other genetic systems involved in the degradation of toxic compounds, such as the synergist effect of a pathway substrate and intermediate in benzoate catabolism (7, 8). In the case of styrene catabolism, the PaaX repressor would modulate StySR-induced transcription of the *sty* genes until the level of PA-CoA signals that the pathway can proceed. Conversely, as styrene is degraded and the StySR-mediated induction decreases, the PA-CoA signal would be anticipated to allow sufficient transcriptional activity to complete degradation of intermediates. In addition, this dual signal-responsive system is likely to ensure a quick transcriptional downshift of the *sty* catabolic genes under a number of conditions. These conditions include (i) whether styrene is not efficiently oxidized to PA and some toxic upper pathway intermediates, such as styrene oxide and/or phenylacetaldehyde, could accumulate within the cell and (ii) when other aromatic compounds, such as toluene and ethylbenzene, induce the StySR activator system (2) but are not enzymatically converted to PA.

## ACKNOWLEDGMENTS

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